APPENDIX I. SUPPLEMENTAL MATERIAL

Diminished force production and mitochondrial respiratory deficits are strain-dependent myopathies of subacute limb ischemia.

Short Title: Strain dependent mitochondrial dysfunction in subacute ischemia

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Materials and Methods

Animals. Experiments were conducted on adult (12-16 week) C57BL/6J (N=33) or BALB/cJ (N=36) mice. All work was approved by the Institutional Review Committee of East Carolina University. Animal care was in compliance with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. Washington: National Academy Press, 1996. Sub-acute ischemia was performed as previously described[1], with the following modifications. Mice were anesthetized by intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg) and an ameroid constrictor (AC; 0.25 mm internal diameter; Research Instruments SW, Escondido, Calif) was placed on the femoral artery immediately distal to the lateral circumflex femoral artery and proximal to the origin of the superficial caudal epigastric artery. The inferior epigastric, lateral circumflex, and superficial epigastric artery branches of the femoral artery were left intact to preserve collateral perfusion to the limb. The cardiotoxin model (CTX) of mouse muscle regeneration was performed as previously described[2] using 20μL I.M. injections of 5μM *Naja nigricollis* venom into the tibialis anterior, medial and lateral heads of the gastrocnemius with a 27 gauge needle under anesthesia. An equivalent volume sham injection of 1X phosphate buffered saline (PBS) was administered to the muscles of the contralateral hindlimb. Because CTX was not directly injected into the EDL, but EDL muscles were used for various measurements in this study, a test animal (C57BL/6) was sacrificed 24hrs following TA injection of CTX and the EDL muscle was isolated and histologically stained to confirm that the extent of injury was identical to the TA muscle.

Assessment of limb perfusion and tissue SO_2. Limb blood flow was measured using laser Doppler perfusion (LDPI) imaging as previously described[1] with the following modifications. Imaging was performed at a 4ms/pixel scan rate on animals placed on a 37C warming pad in the prone position under ketamine/xylazine anesthesia, using a Moor Instruments LDI2-High Resolution (830 nM) System (Moor, Axmin- ster, UK) up to 28 days post intervention. Hindlimb hair was removed with depilatory cream 24 hours prior to initial scanning and hair was removed with a microshaver at all other timepoints. Images were analyzed with the MoorLDI Image Review software. Tissue oxygen saturation (SO_2) was assessed at the aforementioned timepoints using a Moor VMS-OXY white light spectrometer with a CPT-300 optical probe. SO_2 measurements were taken immediately following LDPI imaging for each animal to minimize positional variation. The optical probe was placed on the ventro-medial region of the paw (SLI, CTX), as well as the TA and lateral head of the gastrocnemius muscle (CTX). Stable signal was collected for ten seconds. Data were analyzed using the Moor VMS review software. Results for both analyses were expressed as a ratio of the treated limb to the untreated (SLI) or sham injected (CTX) contralateral limb.

Primary Antibodies and histological stains. The following commercial antibodies were used: CD31 (AbdSerotec MCA-1364), dystrophin (Thermo Scientific RB-9024), total oxphos (AbCam 110413), MHC type I (BA-D5, DHSB), MHC type IIa (SC-71, DHSB), MHC type IIb (BF-F3, DHSB), CD11b (AbCam 52478). DAPI mounting medium (VECTOR Laboratories, H-1200) was also used. Histological stains were obtained from Sigma-Aldrich: Mayer's hematoxylin, F8775; Direct red 80 (sirius red), MHS16; Picric acid, P6744; Weigert's hematoxylin, HT1079.

Immunofluorescence (IF) and Histology. 8-µm-thick (12-µm-thick for oil red o) transverse sections from tibialis anterior frozen in liquid nitrogen cooled isopentane in optimum cutting temperature medium (OCT) were cut using a Leica 3050S cryotome and collected on charged slides for staining. For morphological analyses, standard methods for hematoxylin and eosin (H&E), oil red o (intramuscular lipids), and picrosirius red (collagen) histological staining were performed. Analysis of muscle regeneration was performed on whole image transverse sections compiled of 40X tiled H&E images using an Aperio CS2 digital slide analyzer (Leica Biosystems) and the aperio imagescope software (v12.0, default settings). Sections were segregated into regions of interest (ROI) by morphology representative of tissue regeneration. ROIs were traced by a blinded investigator using the following categories: regenerating, intact myofibers with peripheral nuclei and/or small basophilic myofibers with centralized nuclei and low to moderate granulation tissue; non-regenerating, granulation tissue with few to no basophilic myofibers and/or anucleate necrotic fibers. Muscle tissue morphology was presented as a % of the total muscle area including intact fibers and fascicular structure, the total number of myofibers, and the total number of myofibers with centralized nuclei. Analysis of lipid content was performed on 12-µm sections rinsed in 60% ethanol and stained in oil red o (3 volumes of 1g/100mL oil red o in 99% isopropyl alcohol, in two volumes of 1% dextrin) for 20 mins. Slides were then counterstained in mayer's hematoxylin and washed prior to coverslipping with a glycerol gelatin mounting medium at 37°C. 10X brightfield images were obtained and lipid positive staining was quantified by a blinded investigator in Imagej (NIH, v1.49) by thresholding hue, saturation, and brightness colorspaces and measuring the percent area of lipid positive stain within the field of view.

Upper and lower threshold bounds were kept constant among all images analyzed. Collagen deposition was quantified by picrosirius red. Slides were allowed to come to room temperature and stained in weigert's hematoxylin soln for 10 mins and were then rinsed in circulating tap water for 10 mins. Slides were then stained in a working soln of picrosirius red (sirius red .5g/500mL, saturated picric acid 500mL) for one hour and washed in acidified water before dehydration in ethanol, clearance in xylene, and mounting using glycerol gelatin at 37°C. 10X images were taken on an Olympus BX51-P polarized light microscope and collagen deposition was quantified by a blinded investigator in Imagej (NIH, v1.49) by thresholding hue, saturation, and brightness colorspace and measuring the percent area of the field of view occupied by each of two birefringent hue categories: red/orange and yellow/green. Upper and lower bounds for thresholding were kept constant among all images analyzed. To assess the relative distributions of muscle interstitial cells with lipid positive inclusions, transverse sections were prepared as described above with the exception of fixation. The unfixed sections were immunostained for dystrophin as described above for use as a counterstain. Sections were then stained with 1ug/mL BODIPY 493/503 (ThermoFisher) in 1X PBS diluent for 30 mins. Slides were then mounted in Vectashield hard mount medium with DAPI and imaged as described above. Image quantification was performed on representative 10X images by a blinded investigator using ImageJ (NIH, v1.49) by decomposing the images into RBG colorspace, setting appropriate standard threshold value limits, and measuring the percent area of BODIPY positive stain within each image.

Vascular density and morphology IF was performed as previously described[1, 3]. Briefly, transverse sections were allowed to come to room temperature and were

fixed/permeabilized with ice-cold 1:1 acetone/methanol for 10 minutes at 4C. Fixed sections were allowed to air dry for 5 minutes at room temperature (RT) and rehydrated in 1X PBS before blocking in 5% normal goat serum (Sigma) in 1X PBS at RT for 45 min. Slides were then incubated overnight at 4C in a primary antibody solution. Slides were washed three times in 1X PBS at RT and incubated for 1 hour at RT in the dark in a secondary solution containing a 1:250 dilution of Alexa Fluor 488, or 647 conjugated secondary antibodies in blocking solution. Sections were washed in the dark three times for 5 minutes each with 1X PBS at RT, and coverslips were mounted using Vectashield HardSet Mounting Medium with DAPI (Vector Labs H- 1500). Images were captured using a Life Technologies Evos auto FL wide field fluorescence microscope (Thermo Fisher) and analyzed by a blinded investigator using ImageJ (NIH, v1.49). Five 20X images were captured per section from similar topographical regions. Images were decomposed to red-blue-green (RBG) composites, thresholded using the same upper and lower bounds for all images analyzed, and presented as the mean percent $CD31^+$ area per 20X field of view. These measurements were interpreted as being proportional to the density of the capillary bed in the muscle. Myofiber cross sectional areas were quantified by thresholding dystrophin positive signal until segmentation was achieved and analyzing the area of each complete fiber in the field of view using ImageJ (NIH, v1.49). Myofiber areas were measured in microns squared (μm^2) and presented as a ratio of the insulted limb (L) to the contralateral control limb (R). Muscle fiber type IF staining was performed as previously described[4]. Briefly, transverse sections were allowed to come to room temperature and were fixed/permeabilized with ice-cold 1:1 acetone/methanol for 10 minutes at 4C. Fixed sections were allowed to air dry for 5 minutes at room

temperature (RT) and rehydrated in 1X PBS before blocking in 5% normal goat serum (Sigma) in 1X PBS at RT for 45 min. Slides were then incubated overnight at 4C in a primary antibody solution including antigens directed at dystrophin, MHC type I, MHC type IIa, or MHC type IIb (MHC type IIx would be unstained). Slides were washed three times in 1X PBS at RT and incubated for 1 hour at RT in the dark in a secondary solution containing a 1:250 dilution of Alexa Fluor secondary antibodies in blocking solution. Sections were washed in the dark three times for 5 minutes each with 1X PBS at RT, and coverslips were mounted using Vectashield HardSet Mounting Medium with DAPI (Vector Labs H- 1500). Images were captured using a Life Technologies Evos auto FL wide field fluorescence microscope (Thermo Fisher) and analyzed by a blinded investigator using ImageJ (NIH, v1.49). Four 10X images were captured per section from similar topographical regions using a Life Technologies Evos auto FL wide field fluorescence microscope (Thermo Fisher) and analyzed by a blinded investigator using ImageJ (NIH, v1.49). Data are presented as the percentage of total myofibers of each fiber type (MHC type I, MHC type IIa, or MHC type IIb).

Immunoblotting. Extensor digitorum longus (EDL) muscles were isolated and snap frozen in liquid nitrogen. Frozen muscles were homogenized in ice-cold RIPA Lysis buffer containing protease and phosphatase inhibitors. Protein concentrations were determined using a BCA protein assay (Pierce, ThermoFisher #23225). Proteins were then separated using an SDS-Page gel (Mini-Protean TGX, Bio-Rad #4561093) with 50ug total protein loaded per well. Blots were visualized with chemiluminescence using standard film procedures.

Preparation of isolated skeletal muscle mitochondria. Skeletal muscle mitochondria were isolated from the plantar flexor (i.e. gastrocnemius, plantaris, and soleus) muscles of both control (R) and injured (L) hindlimbs as previously described[5]. Muscle was pooled from two animals to ensure sufficient mitochondrial yield was obtained. Following dissection, muscle was washed in mitochondrial isolation medium (MIM) containing 300mM sucrose, 10mM HEPES, and 1mM EGTA. Muscle was minced on ice using fine tipped scissors for five minutes. Muscle was then washed and resuspended in MIM + 1mg/mL bovine serum albumin (MIM+BSA) and homogenized on ice using a Teflon pestle and Wheaton overhead stirrer. The homogenate was centrifuged at 800xg to pellet non-mitochondrial myofibrillar proteins, nuclei, and other cellular components. The supernatant was transferred to a pre-chilled oakridge tube and then centrifuged at 12,000xg to pellet mitochondria. The mitochondrial pellet was washed and resuspended in 100µl of MIM and stored on ice until analysis (less than 1 hr). Mitochondrial protein content was determined by BCA protein assay (Pierce).

Mitochondrial respiration measurements. High-resolution O_2 consumption measurements were conducted at 37˚C in buffer Z (105 mM K-MES, 30 mM KCl, 1 mM EGTA, 10 mM K_2HPO_4, 5 mM $MgCl_26H_2O$, 0.5 mg/ml BSA, pH 7.1), supplemented with creatine monohydrate (20 mM), using the OROBOROS O2K Oxygraph. A substrate inhibitor titration protocol was performed as follows: 2mM Malate + 10mM Glutamate (State 2 respiration), followed by the addition of 4mM ADP to initiate State 3 respiration supported by Complex I substrates, convergent electron flow through complexes I and II was initiated with the addition of 10mM Succinate, 10µM Rotenone was subsequently added to inhibit Complex I, followed by 10µM Cytochrome C to test the integrity of the

mitochondrial membrane, Complex IV supported respiration was examined using the electron donor N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) at 0.5mM in the presence of 2mM Ascorbate (to limit auto-oxidation of TMPD) and 5μM of Antimycin A (to prevent reverse electron flow through Complex III), finally, uncoupled respiration was assessed with the addition of 0.5μM Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP). The rate of respiration was expressed as pmol/s/mg mitochondrial protein.

Preparation of permeabilized muscle fibers. During sacrifice, a portion of the red gastrocnemius muscle was removed and immediately placed in ice-cold buffer X (50 mM K-MES, 7.23 mM K_2EGTA, 2.77 mM CaK_2EGTA, 20 mM imidazole, 20 mM taurine, 5.7 mM ATP, 14.3 mM phosphocreatine, and 6.56 mM $MgCl_2$-$6H_2O$, pH 7.1) for preparation of permeabilized fiber bundles (PmFBs) as previously described[6]. Fiber bundles were separated along their longitudinal axis using needle-tipped forceps under magnification (MX6 Stereoscope, Leica Microsystems, Buffalo Grove, IL, USA), permeabilized with saponin (30 μg/ml) for 30 minutes at 4°C, and then washed in cold buffer Z (105 mM K-MES, 30 mM KCl, 1 mM EGTA, 10 mM K_2HPO_4, 5 mM $MgCl_2$-$6H_2O$, 0.5 mg/ml BSA, pH 7.4) for approx. 20 minutes until analysis. At the conclusion of each experiment, PmFBs were washed in double-distilled H_2O to remove salts, freeze-dried (Labconco), and weighed. Typical fiber bundle sizes were 0.2-0.4 mg dry weight.

Isolated myofiber respiration measurements. High-resolution O_2 consumption measurements were conducted at 37˚C in buffer Z (105 mM K-MES, 30 mM KCl, 1 mM EGTA, 10 mM K_2HPO_4, 5 mM $MgCl_2 6H_2O$, 0.5 mg/ml BSA, pH 7.1), supplemented with creatine monohydrate (20 mM), using the OROBOROS O2K Oxygraph. An

abbreviated protocol similar to that used for isolated mitochondria was performed, described as follows: 2mM Malate + 10mM Glutamate (State 2 respiration), followed by the addition of 4mM ADP to initiate State 3 respiration supported by Complex I substrates, convergent electron flow was next initiated with the addition of 10mM Succinate, 10μM Rotenone was subsequently added to inhibit Complex I, followed by 10μM Cytochrome C to test the integrity of the mitochondrial membrane. The rate of respiration was normalized to the myofiber dry weight and expressed as pmol/s/mg dry weight.

Mitochondrial calcium retention capacity. To determine susceptibility to opening of the mitochondrial permeability transition pore (mPTP), preparations of isolated mitochondria were exposed to progressively increasing calcium load in the presence of (in mM): 5 malate, 10 glutamate, 0.02 ADP. Changes in extramitochondrial calcium concentration were monitored fluorometrically using Calcium Green (1 μM, excitation/emission 506/532 nm, Invitrogen) per the manufacturer's instructions. All experiments were run at 37 °C in Buffer Z containing 2 U/ml hexokinase and 5 mM 2-deoxyglucose (to clamp respiration).

Citrate synthase activity assays. Activity assays were performed using a citrate synthase activity assay kit (Sigma). Briefly, extensor digitorum longus (EDL) muscles were isolated and snap frozen in liquid nitrogen. Frozen muscles were homogenized in ice-cold Lysis buffer. Protein concentrations were determined using a BCA protein assay (Pierce, ThermoFisher #23225). Activity assays were performed in assay buffer containing (in mM): 100 Tris, 1 EDTA, 1 EGTA, 10 DTNB (Sigma: D8130), and 30 Acetyl CoA at pH 8.35. All samples were measured in triplicate and the average absorbance was used in

final calculations of activity. Background absorbance was measured prior to addition of 10mM oxoloacetate (Sigma: 04126) and final activity rates were corrected for those values.

Muscle contractile force measurements. Contractile force was performed as previously described[7]. In brief, single EDL muscles were surgically excised with ligatures at each tendon (5–0 silk suture) and mounted in a bath between a fixed post and force transducer (Aurora 300B-LR) operated in isometric mode. The muscle was maintained in modified Kreb's buffer solution (PSS; pH 7.2) containing (in mM) 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 2.15 Na_2HPO_4, .85 NaH_2PO_4, and maintained at 25°C under aeration with 95% O_2–5% CO_2 throughout the experiment. Resting tension and muscle length were iteratively adjusted for each muscle to obtain the optimal twitch force and a supramaximal stimulation current of 600mA was used for stimulation. After a 5 min equilibration, isometric tension was evaluated by 200 ms trains of pulses delivered at 10, 20, 40, 60, 80, 100, and 120 Hz. Length was determined with a digital microcaliper. After the experimental protocol, muscles were trimmed proximal to the suture connections, excess moisture was removed, and the muscle was weighed. The cross-sectional area for each muscle was determined by dividing the mass of the muscle (g) by the product of its length (L_0, mm) and the density of muscle (1.06 g cm^{-3}) and was expressed as millimeters squared (mm^2). Muscle output was then expressed as specific force (N/cm^2) determined by dividing the tension (N) by the muscle cross-sectional area.

Total RNA and qRT-PCR Gene Expression. Total RNA was extracted from mouse EDL muscles using TRIzol (Invitrogen) phenol/chloroform extraction. RNA was reverse-transcribed using SuperScript IV Reverse Transcriptase and random primers (Invitrogen).

Reactions were incubated at 50°C for 50 minutes and at 85°C for 5 minutes. Real-time PCR was performed using an ABI ViiA-7 system (Applied Biosystems). Relative quantification of MyoD Family Inhibitor (MDFI), MAFbx, Murf-1, TNF **α, IL-1 β, IL-6** mRNA levels were determined using the comparative cycles to threshold (ΔΔCT) method using FAM TaqMan gene expression assays (ThermoFisher) specific for each of these genes run in complex (multiplex) with a VIC-labeled 18-S ribosomal subunit control primer.

Statistics. Data are presented as a ratio of the ischemic (L) to the non-ischemic (R) limb, mean ± SEM. Force frequency and fiber typing data are presented as ischemic (L) and non-ischemic (R) limbs for each strain, mean ± SEM. Statistical analyses were carried out using StatPlus:mac (v. 2009), Vassarstats (www.vassarstats.net) or Prism 6 (v. 6.0d) software. *a priori* two sided t-tests were performed to examine mean differences in the control limbs of the strains. All other data were compared using ANOVA with Holm-Sidak multiple comparison's test or uncorrected Student's 2-tailed *t*-test. In all cases, $P<0.05$ was considered statistically significant.

Supplemental References

1. McClung JM, McCord TJ, Southerland K, Schmidt CA, Padgett ME, Ryan TE, et al. Subacute limb ischemia induces skeletal muscle injury in genetically susceptible mice independent of vascular density. J Vasc Surg. 2015.

2. Yan Z, Choi S, Liu X, Zhang M, Schageman JJ, Lee SY, et al. Highly coordinated gene regulation in mouse skeletal muscle regeneration. The Journal of biological chemistry. 2003;278(10):8826-36.

3. McClung JM, McCord TJ, Keum S, Johnson S, Annex BH, Marchuk DA, et al. Skeletal muscle-specific genetic determinants contribute to the differential strain-dependent effects of hindlimb ischemia in mice. The American journal of pathology. 2012;180(5):2156-69.

4. McClung JM, Van Gammeren D, Whidden MA, Falk DJ, Kavazis AN, Hudson MB, et al. Apocynin attenuates diaphragm oxidative stress and protease activation during prolonged mechanical ventilation. Crit Care Med. 2009;37(4):1373-9.

5. Lark DS, Reese LR, Ryan TE, Torres MJ, Smith CD, Lin CT, et al. Protein Kinase A Governs Oxidative Phosphorylation Kinetics and Oxidant Emitting Potential at Complex I. Frontiers in physiology. 2015;6:332.

6. Perry CG, Kane DA, Lin CT, Kozy R, Cathey BL, Lark DS, et al. Inhibiting myosin-ATPase reveals a dynamic range of mitochondrial respiratory control in skeletal muscle. Biochem J. 2011;437(2):215-22.

7. Spangenburg EE, Le Roith D, Ward CW, Bodine SC. A functional insulin-like growth factor receptor is not necessary for load-induced skeletal muscle hypertrophy. J Physiol. 2008;586(1):283-91.

Supplemental Figure Legends

Supplemental Figure 1. Strain dependent myopathy with subacute hindlimb ischemia. Sub-acute femoral artery occlusion was performed on C57BL/6 and BALB/c mice by placement of a single AC (1AC) on the proximal portion of the femoral artery, immediately proximal to the epigastric arterial branch. **A.** Representative 2X and 20X images of hematoxylin and eosin (H&E) stained sections of tibialis anterior (TA) muscles subjected to 7 and 28 days of sub-acute ischemia. R, contralateral control limb; L, ischemic limb. Arrows indicate intact muscle; Chevrons indicate regions of anucleate necrotic fibers. Scale bar inlays are 1000μm in length(2X), and 200μm in length(20X). **B**. Muscle regeneration was quantified from 2X H&E images by measuring the total regions of interest (ROI; area) containing necrotic/anuclueate myofibers and subtracting from total muscle area to give a percent area of intact muscle, which is represented by median and interquartile range. **C**. Total myofibers with centralized nuclei (Central. Nuclei) and total myofiber number (Intact Fibers) were determined in representative 10X images. Data are represented by mean ± SEM. All data are representative of N=6/strain/timepoint for each observation. * $P<0.05$ vs. day matched C57BL/6.

Supplemental Figure 2. Atrophy associated gene expression in ischemic hindlimbs. Sub-acute femoral artery occlusion was performed on C57BL/6 and BALB/c mice by placement of a single AC (1AC) on the proximal portion of the femoral artery. QRT-PCR was utilized to determine extensor digitorum longus (EDL) mRNA expression of MAFbx (**A**, muscle atrophy F-box), MDFI (**B**, MyoD family inhibitor 1), and MuRF-1 (**C**, muscle ring finger protein-1). Data are representative of corrected values for ribosomal subunit

18S mRNA and expressed as a ratio of the ischemic (L) to the non-ischemic (R) limb, mean ± SEM. N=6/strain/timepoint for each observation. * $P<0.05$ vs. day matched C57BL/6.

Supplemental Figure 3 Non-myofiber tissue deposition in ischemic hindlimb muscle. Sub-acute femoral artery occlusion was performed on C57BL/6 and BALB/c mice by placement of a single AC (1AC) on the proximal portion of the femoral artery. A. Sections of TA muscles were stained with picrosirius red dye (PR, birefringent dye) to determine collagen deposition. Representative 10X images of PR taken through a polarized light filter at 7 and 28 postoperative days. Arrows indicate regions containing thick collagen (orange/red birefringence); Chevrons indicate regions containing thin collagen (green/yellow birefringence). Scale bar inlays are 400μm in length. B. Green/yellow hue distribution (thin collagen deposition). C. Orange/red hue distribution (thick collagen deposition). D. Sections of TA muscles were immunofluorescently labeled with $CD31^+$ ($PECAM\text{-}1^+$) for the analysis of capillary density. Scale bar inlays are 200μm in length (E). All data are presented as the ratio of the ischemic (L) to the non-ischemic (R) limb, mean ± SEM and representative of N≥6/strain/time point. * $P=0.06$ vs. day matched C57BL/6.

Supplemental Figure 4. Strain dependent intramuscular lipid content during ischemia. Sub-acute femoral artery occlusion was performed on C57BL/6 and BALB/c mice by placement of a single AC (1AC) on the proximal portion of the femoral artery. **A**. Sections of TA muscle were histologically stained with Oil Red-O to determine

muscle lipid content. Scale bar inlays are 400μm in length. (**B**). **C**. Sections of TA muscles were also immunofluorescently labeled with BODIPY for the determination of intermuscular lipid inclusions. Scale bar inlays are 400μm in length. (**D**). All data are presented as ratio of the ischemic (L) to the non-ischemic (R) limb, mean ± SEM and representative of N=6/strain/timepoint for each observation. Non-significant trend observed at 28d for Oil Red-O (p=0.09).

Supplemental Figure 5 Skeletal muscle innate immune response during ischemia. Sub-acute femoral artery occlusion was performed on C57BL/6 and BALB/c mice by placement of a single AC (1AC) on the proximal portion of the femoral artery. Western blotting was performed for qualitative observation of Mac-1 (CD11b) protein abundances, with GAPDH protein blotting as a loading reference (**A,B**). QRT-PCR was utilized to determine extensor digitorum longus (EDL) mRNA expression of TNF-α (**C**, Tumor necrosis factor-alpha), IL-1β (**D**, Interleukin 1 beta), and IL-6 (**E**, Interleukin 6). Data are representative of corrected values for ribosomal 18S subunit mRNA and expressed as a ratio of the ischemic (L) to the non-ischemic (R) limb, mean ± SEM. N=6/strain/timepoint for each observation. * P<0.05 vs. day matched C57BL/6

Supplemental Figure 6. Verification of myotoxin injury as non-ischemic. Cardiotoxin (CTX) was injected intramuscularly into the TA and the lateral/medial heads of the gastrocnemius muscle. A sham injection of equal volume sterile saline was injected in the same muscles in the contralateral control limb (R). **A**. Laser Doppler perfusion imaging (LDPI) was performed and regionally analyzed (thigh, paw, and whole limb). **B**. Tissue

oxygen saturation (SO_2) was measured via white light reflectance spectroscopy over the tibialis anterior and Gastrocnemius muscles after injury. Data are representative of the ratio of the ischemic (L) to the non-ischemic (R) limb, mean ± SEM. All data are representative of N=6/strain/timepoint for each observation. * $P<0.05$ vs. day matched C57BL/6.

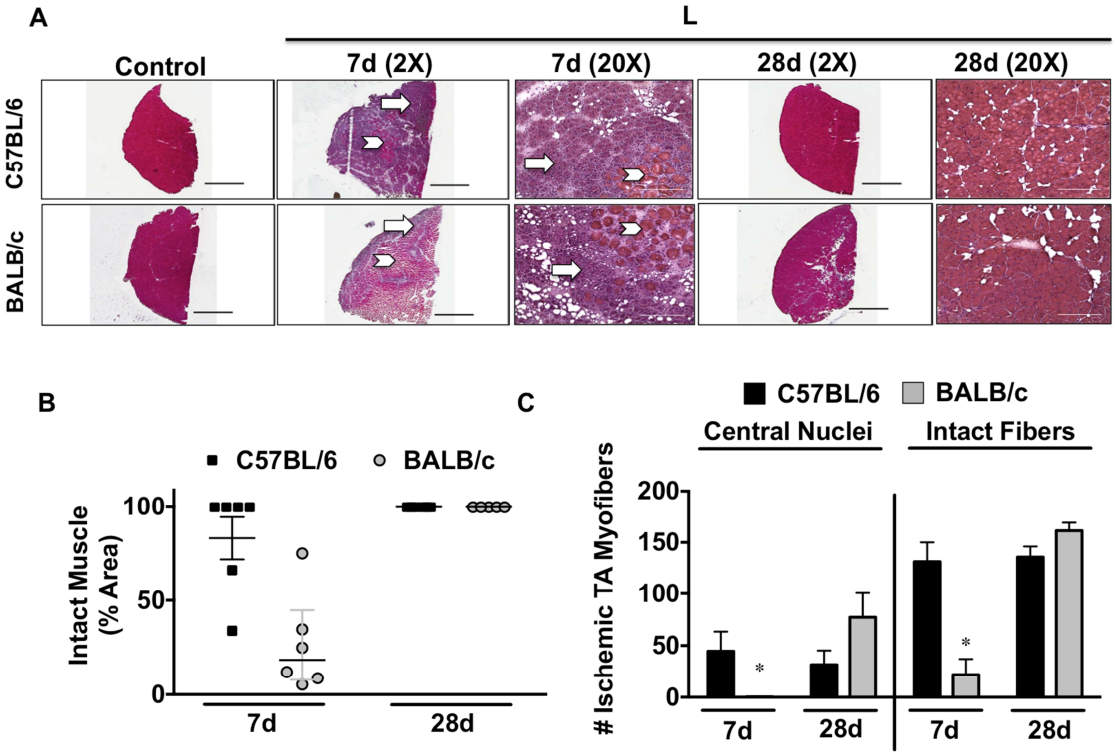
Supplemental Figure 7. Distribution of myotoxic injury to the anterior limb compartment. Cardiotoxin (CTX) was injected intramuscularly into the TA and the lateral/medial heads of the gastrocnemius muscle. Representative 20X images of hematoxylin and eosin (H&E) stained sections of tibialis anterior (TA) and extensor digitorum longus (EDL) muscles 24hrs after CTX injection into the TA. R, contralateral control limb; L, ischemic limb.

Supplemental Figure 8. Non-myofiber tissue deposition in non-ischemic regenerating muscle. Non-ischemic muscle regeneration was induced by cardiotoxin (CTX) injection. **A**. Sections of TA muscles were stained with picrosirius red dye (PR, birefringent dye) to determine collagen deposition. Representative 10X images of PR taken through a polarized light filter at 7 and 28 postoperative days. Arrows indicate regions containing thick collagen (orange/red birefringence); Chevrons indicate regions containing thin collagen (green/yellow birefringence). Scale bar inlays are 400μm in length **B**. Green/yellow hue distribution (thin collagen deposition). **C**. Orange/red hue distribution (thick collagen deposition). **D**. Sections of TA muscle were immunofluorescently labeled with $CD31^+$ ($PECAM\text{-}1^+$) for the analysis of capillary

density (**E**). Scale bar inlays are 200μm in length. All data are presented as ratio of the ischemic (L) to the non-ischemic (R) limb, mean ± SEM and representative of N≥6/strain/time point. * $P<0.05$ vs. day matched C57BL/6, or indicated non-significant trend vs. day matched C57BL/6 Thin Collagen ($p=0.07$).

Supplemental Figure 9. Strain dependent intramuscular lipid content during non-ischemic injury. Cardiotoxin (CTX) was injected intramuscularly into the TA and the lateral/medial heads of the gastrocnemius muscle. **A**. Sections of TA muscle were histologically stained with Oil Red-O to determine muscle lipid content. Scale bar inlays are 400μm in length (**B**). **C**. Sections of TA muscles were also fluorescently labeled with BODIPY for the determination of intermuscular lipid inclusions. Scale bar inlays are 400μm in length (**D**). All data are presented as ratio of the ischemic (L) to the non-ischemic (R) limb, mean ± SEM and representative of N=6/strain/timepoint for each observation. * Non-significant trend observed at 7d for Oil Red-O ($p=0.07$).

Supplemental Figure 1



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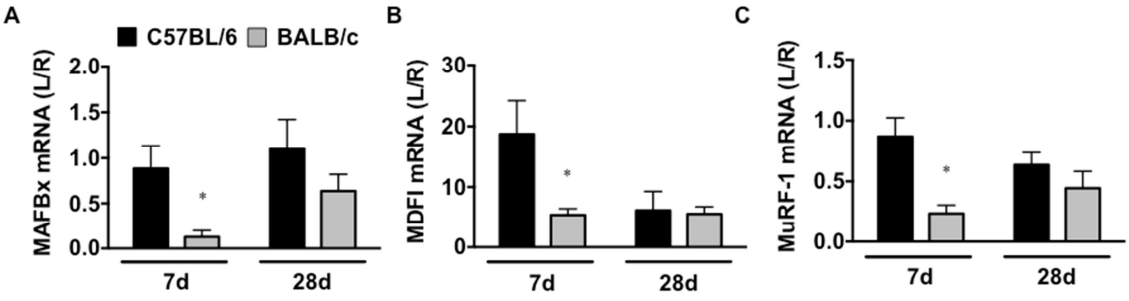

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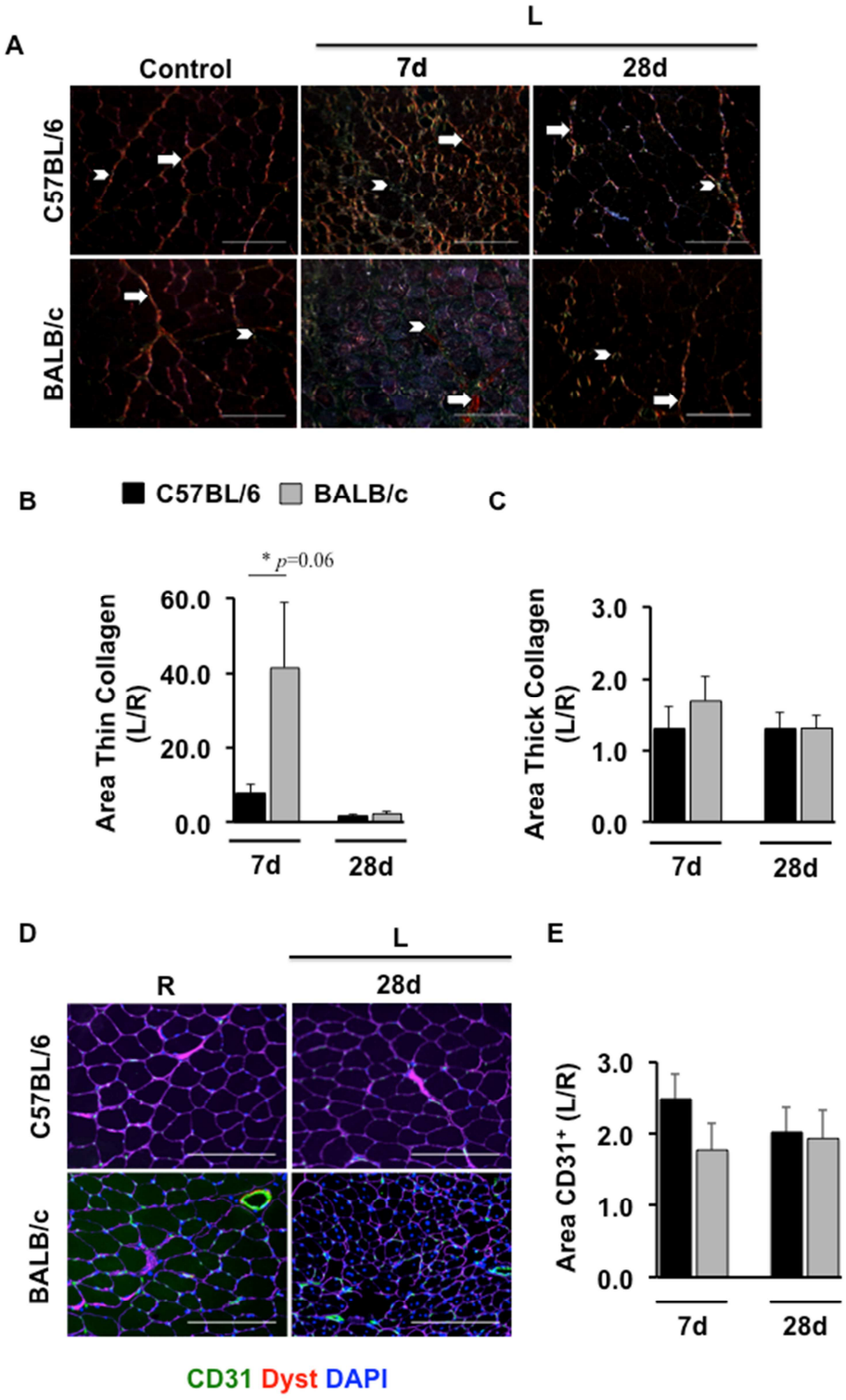

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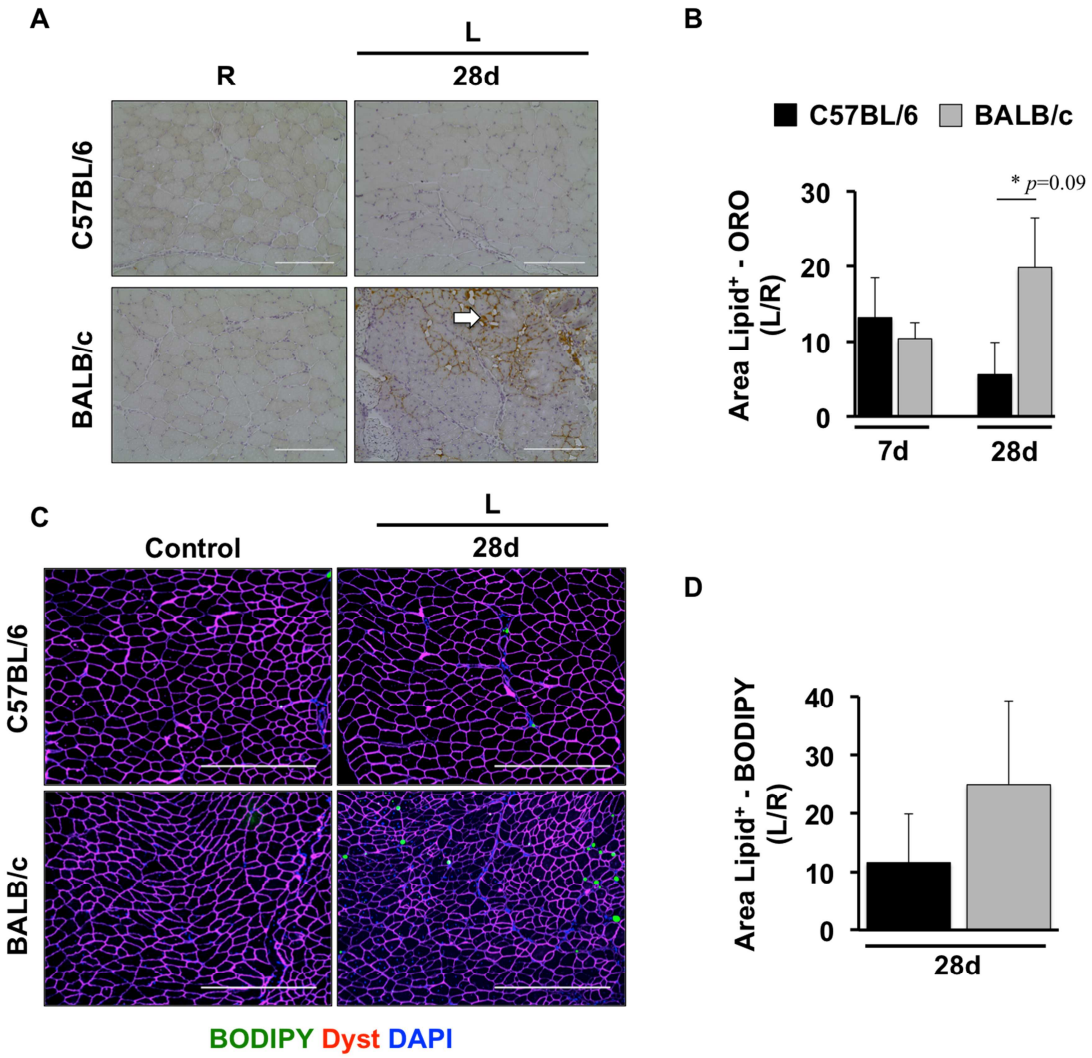
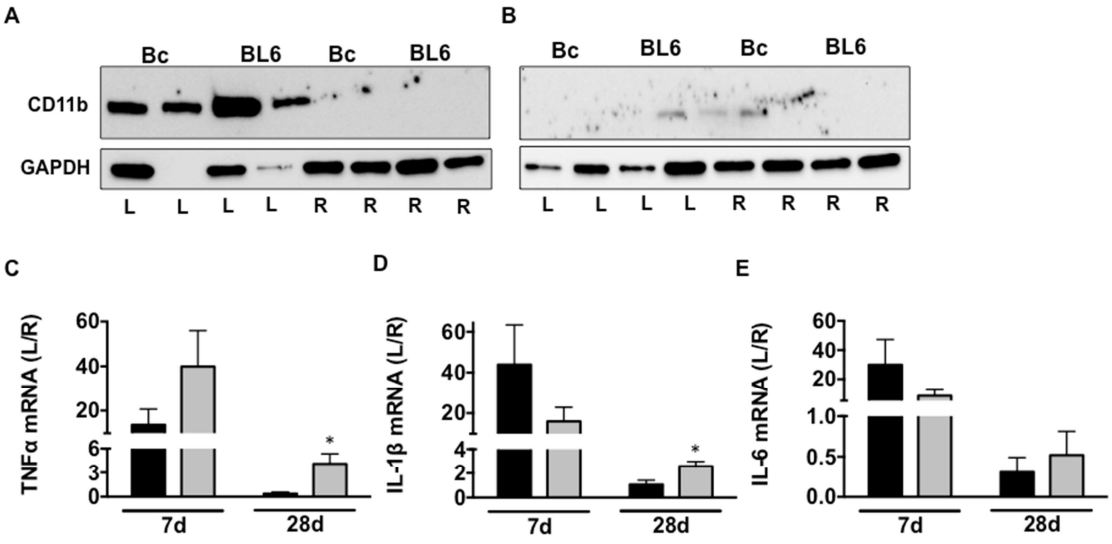
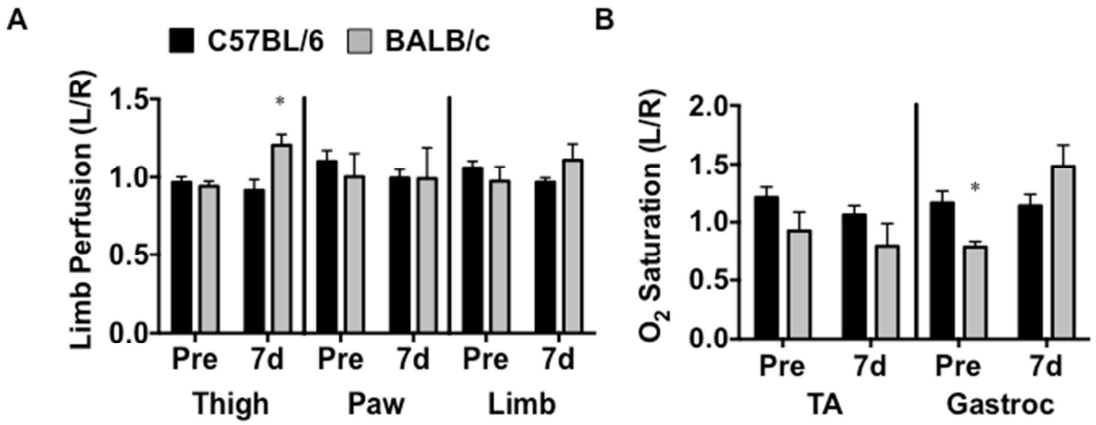


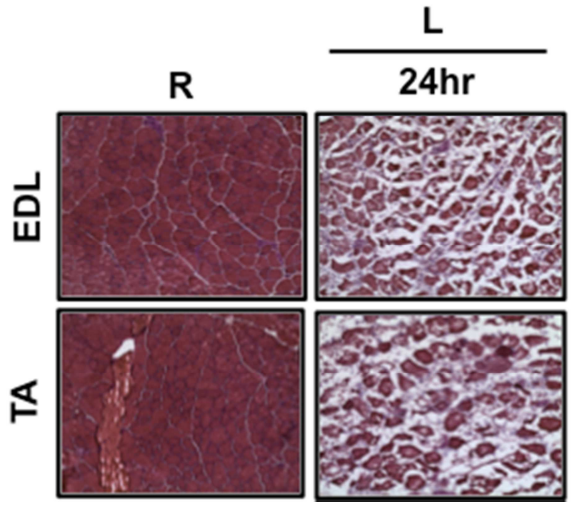
Supplemental Figure 5



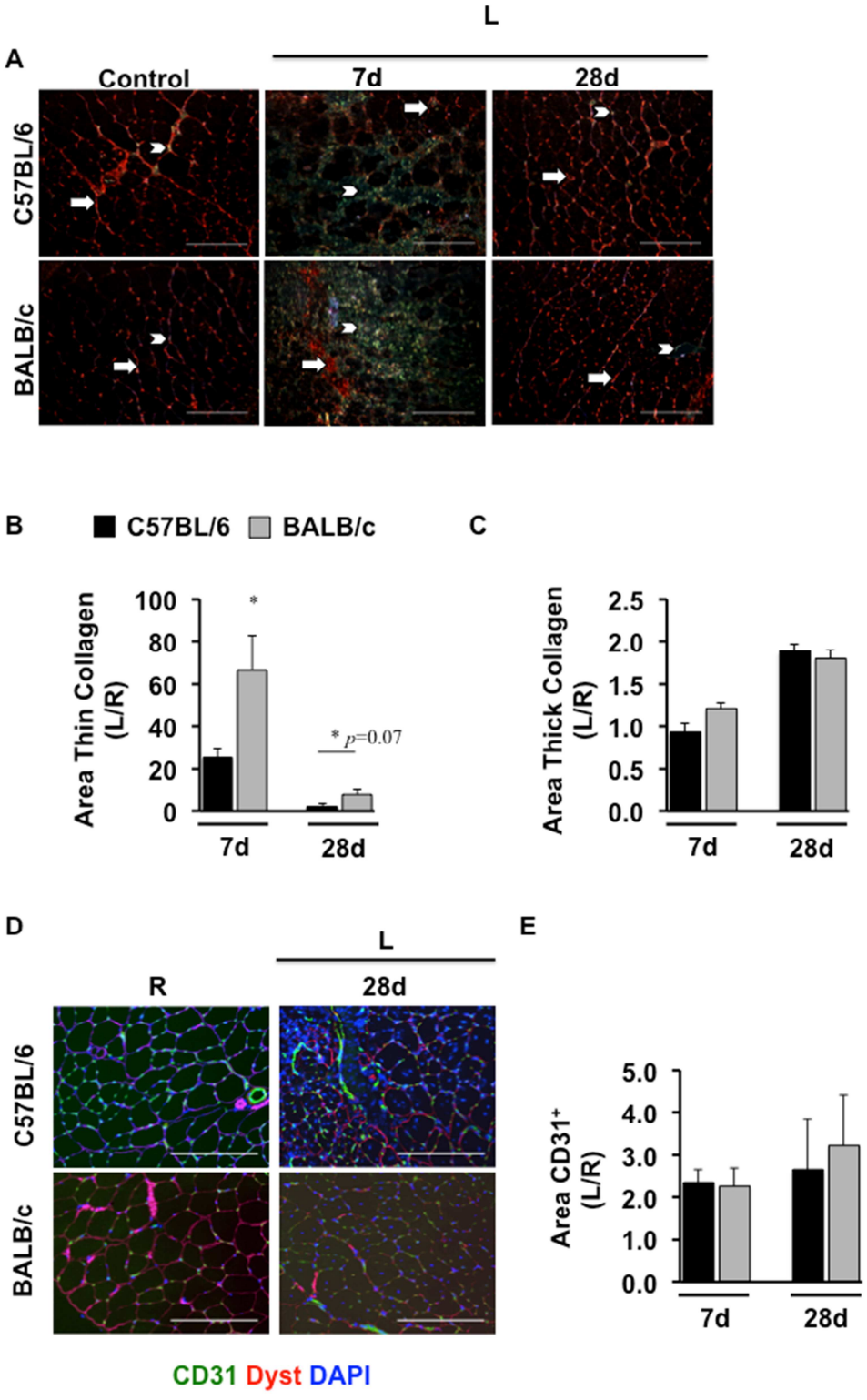
Supplemental Figure 6



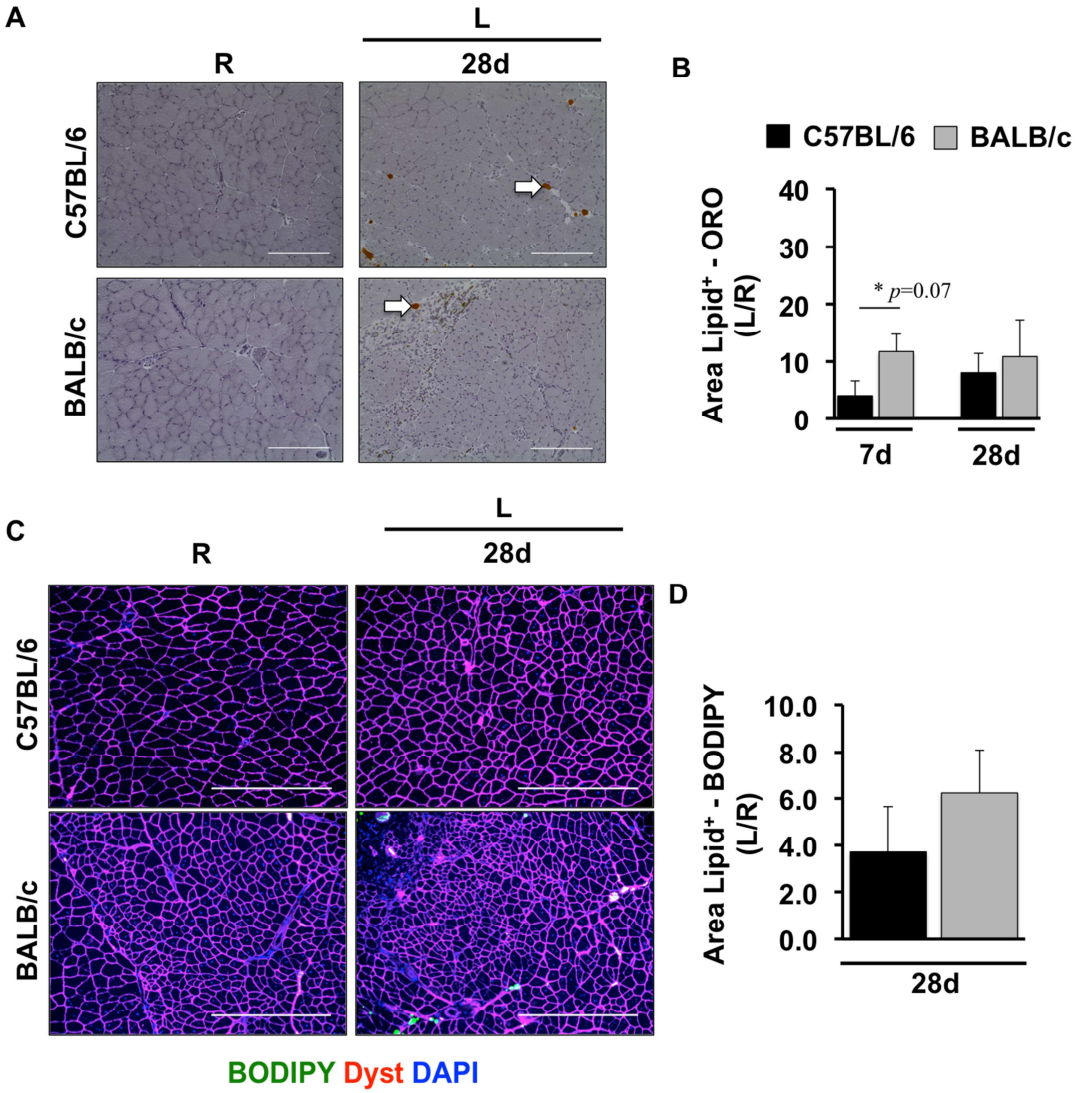
Supplemental Figure 7



Supplemental Figure 8



509 **Supplemental Figure 9**



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